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Disturbances in *ex vivo* vascular smooth muscle responses following exposure to *Pasteurella haemolytica* vaccines

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Weekley, L.B., Eyre, P. Disturbances in *ex vivo* vascular smooth muscle responses following exposure to *Pasturella haemolytica* vaccines. *J. vet. Pharmacol. Therap.* **16**, 446–453.

Rats were vaccinated with saline (control) or one of the two commercially available *Pasteurella haemolytica* vaccines Presponse or Precon-PH. Animals were killed 3 days later and thoracic aorta removed for evaluation of the *ex vivo* biophysical responses to carbachol (CCh). In some experiments, vascular endothelium was mechanically removed. Vaccination of rats impairs the endothelial-dependent relaxation to CCh. In vessels with endothelium removed, the contractile response to CCh is converted into a relaxation following vaccination. Treatment of endothelial-denuded vascular rings *ex vivo* with methylene blue, a guanylate cyclase inhibitor, reduced the vaccination effect. Treatment of vascular rings with the superoxide dismutase inhibitor diethyldithiocarbamate, impairs the relaxant reponse of de-endothelialized vessels to CCh in Presponse vaccinated rats while enhancing the relaxation response of vessels from Precon-PH vaccinated rats. De-endothelialized vessels from vaccinated rats, but not control rats, relaxed in the presence of *N*-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of nitric oxide synthetase. Furthermore, in the presence of L-NMMA, the relaxant response to CCh is significantly enhanced by Precon-PH but not Presponse. The normal relaxant response to hydrogen peroxide is converted into a contraction following vaccination. Results suggest that exposure to commercially available *P. haemolytica* vaccines alters vascular smooth muscle reactivity to CCh and that several independent pathways may be altered.

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INTRODUCTION

Acetylcholine plays a role in regulating vascular smooth muscle tone by a direct action on vascular smooth muscle and indirectly through the release of mediators such as endothelium derived relaxing factor (EDRF; nitric oxide) and oxygen centred free radicals (Furchgott & Zawadski, 1980; Varani *et al.*, 1985). Bovine pneumonic pasteurellosis (BPP), a cranioventral fibrinous bronchopneumonia, is usually associated with vascular damage and thrombi (Breider *et al.*, 1988). *Pasteurella haemolytica*, the pathogen most commonly associated with BPP, has been shown to contain virulence factors which

damage pulmonary artery endothelial cells *in vitro* (Breider *et al.*, 1990). Furthermore, intravenous injection of *P. haemolytica* is capable of causing haematogenous pneumonia (Thomas *et al.*, 1989).

Vaccination against pasteurellosis has had limited efficacy in preventing development of BPP (Martin, 1983). Management often includes stressful practices during the immediate post-vaccination period and subsequent development of disease has been related by some authors to such practices (Dyer, 1982; Shoo, 1989). Both modified live vaccines (e.g. Precon-PH) as well as *P. haemolytica* leukotoxoid (e.g. Presponse), a cell-free, endotoxin-free product, have been used to vaccinate animals. Since the development of BPP is often associated with a stressful event (e.g. vaccination, transport), it seems reasonable to propose that the host response to vaccination may temporarily impair disease resistance during the post-vaccination period. This study was undertaken to examine the effect that *P. haemolytica* antigens derived from commercial vaccines have on cholinergic responses of vascular smooth muscle during the immediate (3 days) post-vaccination period.

METHODS

Animals and drug treatment

Male Sprague–Dawley rats (150–175 g) were used in these studies. Animals were housed at 22 ± 3°C in stainless steel hanging cages (two rats per cage) on a 12L:12D photoperiod. Animals were offered tap water and Purina Rat Chow *ad libitum*. Following a 2-week acclimation period, rats were vaccinated (i.p.) with 1.0 ml Presponse (American Cyanamid, Wayne, NJ, USA), 0.5 ml Precon-PH (A. H. Robins, Richmond, VA, USA; which contained 1.5×10^5 live *P. haemolytica* per ml) or saline. Three days later, rats were killed by an overdose of sodium pentobarbital (80 mg/kg, intraperitoneally (i.p.)) containing 25 U heparin sodium. The 3-day post-vaccination time point was chosen since stressful (e.g. transport) events occurring during the immediate post-vaccination period are often associated with the subsequent development of BPP.

Tissue preparation

Aorta was very carefully removed (avoiding damage to the endothelium) from the dorsal thoracic region and placed in ice-cold (4°C) Krebs–Henseleit solution which had been saturated with 95% O_2:5% CO_2. Blood was washed from the lumen and in some vessels endothelium removed by inserting a glass rod into the lumen and gently rolling (Furchgott & Zawadski, 1980). Vascular rings were placed (37°C) under 2 g initial resting tension and spontaneous tone allowed to develop over the next 2 h.

Ex vivo *drug treatment of vascular rings*

Following the equilibration period, vascular rings were treated with cumulative half or full log dose increments of carbachol (CCh) over the range from 10^{-9} M to 10^{-4} M. Changes in tension (millinewtons; mN) were determined and plotted against the drug concentration. In some experiments, vascular rings (in which the endothelium had been removed) from control or vaccinated rats were pretreated with 10 μM methylene blue (MeB; Fisher Scientific, Fairlawn, NJ, USA), a guanylate cyclase inhibitor, 50 μM L-N^G-monomethyl-L-arginine (L-NMMA; Calbiochem, La-Jolla, CA, USA), a competitive inhibitor of nitric oxide synthetase, or 5.0 mM diethyldithiocarbamate (DETC; Sigma Chemical Co., St Louis, MO, USA), an inhibitor of superoxide dismutase, for 30 min prior to CCh. In separate experiments, vascular rings (in which the endothelium had been removed) from control or vaccinated rats were exposed to 1.0 μM dibutyryl cyclic guanosine monophosphate (Sigma Chemical Co.), 0.1 mM L-arginine (Sigma Chemical Co.), 50 μM L-NMMA (Calbiochem) or 0.3% hydrogen peroxide (Fisher Chemical Co.) and the change in tension from baseline monitored. In all experiments, the new baseline tension had stabilized within 30 min.

Measurement of smooth muscle responses

Smooth muscle isometric contractile responses were measured with a four-channel

analogue transducer manifold model TBM-4 (World Precision Instruments, New Haven, CT, USA). Analogue signals were passed through a Maclab A/D signal converter (World Precision Instruments) and the smooth muscle responses displayed on a model M5011 MacIntosh SE Computer. Records of vascular smooth muscle responses were stored on a 3.5 in. computer disk.

Analysis of data

Aortic ring contractile or relaxation responses were characterized by integrating the change in tension developed by rings in response to cumulative doses of CCh, i.e. mN tension versus the $\log_{10}$ of the molar concentration of drug. The threshold of a tension change to CCh was determined by the x-intercept of the dose–response curve for each vascular ring using points within 20–80% of the maximum response. pD_2 values ($-\log EC_{50}$, where EC_{50} = concentration of CCh causing a half maximal relaxation or contraction) were calculated by linear regression after semi-logarithmic transformation of dose responses. Tests for differences between calculated threshold and pD_2 values were based on mean log values. The influence of drugs on vascular ring tension was examined by a one-way analysis of variance (ANOVA). Individual points were compared by Student's *t*-test. Differences with probabilities of 0.05 or less were accepted as significant. All data are expressed as the mean ± SEM.

RESULTS

Saline vaccination

In aorta with endothelium intact, CCh caused a dose-dependent relaxation with a calculated threshold of 2.0 ± 0.66 nM CCh, pD_2 of 6.65 ± 0.18 M CCh and maximum relaxation of 5.89 ± 0.64 mN (Fig. 1).

In vessels from saline vaccinated rats with endothelium removed, vascular rings contract in response to CCh. This contractile response has a calculated threshold of 5.0 ± 0.14 nM CCh, pD_2 of 6.9 ± 0.42 M CCh and maximum tension of 2.2 ± 0.86 mN (Fig. 2). Preincubation of endothelial-denuded vascular rings from control animals with L-NMMA converts the CCh-mediated contraction into a slight relaxation with a calculated threshold of 0.06 ± 0.47 nM CCh, pD_2 of 7.69 ± 0.14 M CCh and maximum relaxation of 1.67 ± 0.25 mN (data not shown). Preincubation of endo-

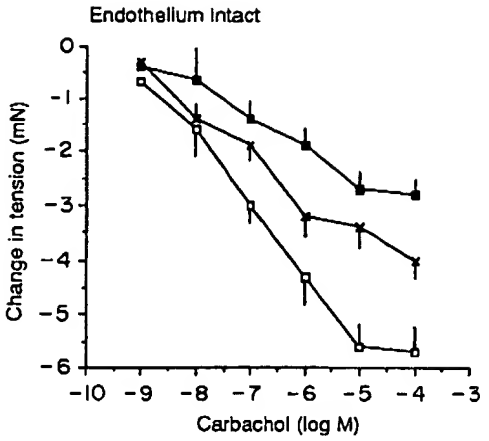

FIG. 1. CCh-mediated relaxant responses of endothelial-intact aortic vascular rings *ex vivo* 3 days following vaccination. Each point is expressed as mN tension change and presented as the mean ± SEM (n = 5–7). —□—, control; —■—, Precon; —*—, Presponse.

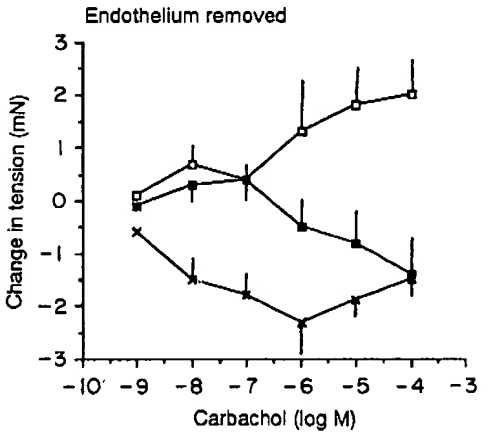

FIG. 2. CCh-mediated changes in endothelial-denuded aortic vascular ring tension *ex vivo* 3 days following vaccination. Each point is expresed as mN tension change and presented as the mean ± SEM (n = 3–9). —□—, control; —■—, Precon; —*— Presponse.

thelial-denuded vascular rings from control animals with DETC eliminated biophysical responses to CCh (data not shown).

In separate experiments (n = 3–4), exposure of endothelial-denuded vessels to 1.0 μM dibutyryl cyclic GMP, 1 mM *l*-arginine, 50 μM L-NMMA or 0.3% hydrogen peroxide caused relaxations of 0.94 ± 0.16, 0.83 ± 0.12, 0.25 ± 0.22, and 4.60 ± 1.30 mN respectively.

Presponse vaccination

In aorta with endothelium intact, CCh caused a dose-dependent relaxation (Fig. 1) with a calculated threshold of 0.81 ± 0.56 nM CCh, pD_2 of 6.94 ± 0.14 M CCh and maximum relaxation of 4.00 ± 0.50 mN ($P < 0.06$ *vs* controls).

In vessels from Presponse vaccinated rats with endothelium removed, vascular rings relax in response to CCh. This relaxant response has a calculated threshold of 1.0 ± 0.26 nM CCh, pD_2 of 7.37 ± 0.33 M CCh and maximum relaxation response of 2.6 ± 0.70 mN (Fig. 2). This relaxant response is in contrast to the contractile response in vessels from saline injected rats. Preincubation of endothelial-denuded vessels from Presponse vaccinated rats with L-NMMA significantly shifted the calculated threshold (0.025 ± 0.45 nM CCh) to the left without altering the pD_2 (7.48 ± 0.04 M CCh) or maximum relaxation (4.01 ± 0.75 mN) attained as compared to vessels from Presponse vaccinated rats not preincubated with L-NMMA (data not shown). Preincubation of endothelial-denuded vacular rings from Presponse vaccinated rats with DETC eliminates biophysical responses to CCh (Fig. 3). Preincubation of endothelial-denuded vascular rings from Presponse vaccinated animals with MeB did not alter the calculated threshold (2.0 ± 0.38 nM CCh) or maximum relaxation attained (1.70 ± 0.31 mN) while the pD_2 is shifted to the right (6.15 ± 0.26 M CCh) (Fig. 4).

In separate experiments (n = 3–4), exposure of endothelial-denuded vessels from Presponse vaccinated rats to 1.0 μM dibutyryl cyclic GMP, 0.1 mM *l*-arginine, 50 μM L-NMMA or 0.3% hydrogen peroxide caused a relaxation of 0.91 ± 0.17 mN, 0.67 ± 0.07 mN, 0.70 ± 0.20 mN (significantly diffferent from controls), and a contraction of 1.32 0.15 mN (significantly different from controls) respectively.

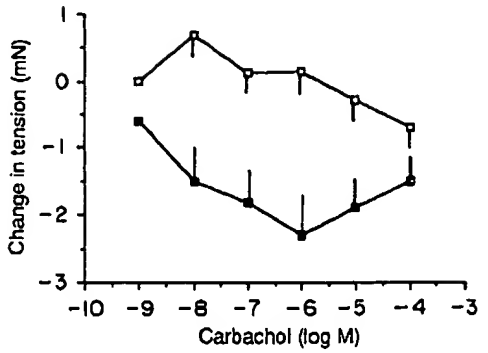

FIG. 3. The effect of DETC (5.0 mM) pretreatment (30 min) *ex vivo* on the tension changes following CCh treatment in Presponse vaccinated rats. Vascular endothelium was removed in these experiments. Each point is expressed as mN tension change and presented as the mean ± SEM (n = 4–9). —■—, Presponse; —□—, Presponse & DETC.

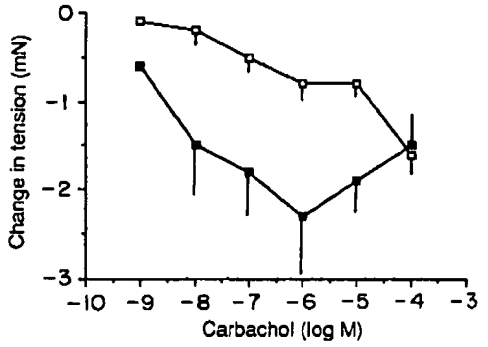

FIG. 4. The effect of MeB pretreatment (10 μM) *ex vivo* on the tension changes following CCh treatment in Presponse vaccinated rats. Vascular endothelium was removed in these experiments. Each point is expressed as mN tension changes and presented as the mean ± SEM (n = 4–9). —■—, Presponse; —□—, Presponse & MeB.

Precon-PH vaccination

In aorta with endothelium intact, CCh caused a dose-dependent relaxation (Fig. 1) with a calculated threshold of 2.0 ± 0.28 nM CCh, pD_2 of 6.74 ± 0.27 M CCh and a maximum relaxation of 2.58 ± 0.53 mN

(significantly different from saline treated with endothelium intact). In vessels from Precon-PH vaccinated rats with endothelium removed, vascular rings relax in response to CCh (Fig. 2). This relaxant response has a calculated threshold of 62.0 ± 0.28 nM CCh, pD_2 of 6.23 ± 0.04 M CCh and a maximum relaxation of 3.48 ± 1.98 mN. This relaxant response is in contrast to the contractile response of endothelial-denuded vessels from saline treated rats. Preincubation of endothelial-denuded vessels from Precon-PH vaccinated rats with L-NMMA caused a significant left shift in the calculated threshold (0.0063 ± 0.12 fM CCh), pD_2 (9.68 ± 0.56 M CCh) and an increase in the maximum relaxation attained (8.59 ± 0.70 mN) as compared to vessels from Precon-PH vaccinates not preincubated with L-NMMA and vessels from saline vaccines preincubated with L-NMMA (Fig. 5). The left shift in calculated threshold is probably somewhat artifactual since it is derived from the slope of the concentration–response curve, although it probably does represent a biologically significant change. Preincubation of vessels from Precon-PH vaccinated rats with DETC causes a relaxation response to CCh with a left shift in the calculated threshold to 0.079 ± 0.66 nM CCh and pD_2 to 7.53 ± 0.32 M CCh as compared to vessels from Precon-PH vaccinates not preincubated with DETC. The maximum relaxation attained (3.35 ± 0.92 mN) is not altered by preincubation with DETC (Fig. 6). This relaxant response to CCh stands in contrast to the elimination of any response in vessels from saline (data not shown) or Presponse (Fig. 3) vaccinated rats. Preincubation of endothelial-denuded vascular rings from Precon-PH vaccinated animals with MeB converted the CCh-mediated relaxant response into a contraction with a calculated threshold of 3.0 ± 0.34 nM CCh, pD_2 of 5.72 ± 0.04 M CCh and maximum contraction of 1.50 ± 0.33 mN (Fig. 7).

In separate experiments (n = 3–4), exposure of endothelial-denuded vessels to 1.0 μM dibutyryl cyclic GMP, 0.1 mM L-arginine, 50 μM L-NMMA or 0.3% hydrogen peroxide caused relaxations of 0.35 ± 0.43, 0.26 ± 0.45, 0.97 ± 0.88 (significantly different from controls) and a contraction of 1.65 ± 0.49 mN (significantly different from controls) respectively.

DISCUSSION

These experiments demonstrate that vaccination with Precon-PH or Presponse vaccines impairs the efficacy of CCh as a relaxant agent in endothelial intact vessels. This impairment is greater in rats vaccinated with Precon-PH, a live bacterial vaccine. Since CCh mediates much of its relaxant properties in endothelial

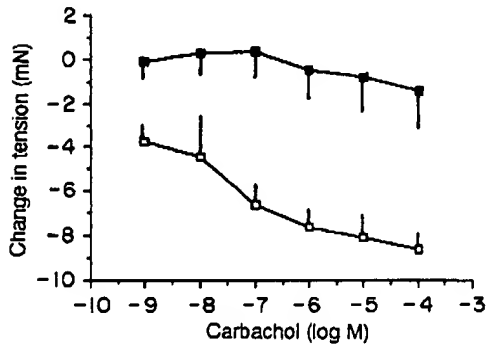

FIG. 5. The effect of L-NMMA pretreatment (50 μM) *ex vivo* on the tension changes following CCh treatment in Precon-PH vaccinated rats. Vascular endothelium was removed in these experiments. Each point is expressed as mN tension change and presented as the mean ± SEM (n = 4–9). —■—, Precon; —■—, Precon & L-NMMA.

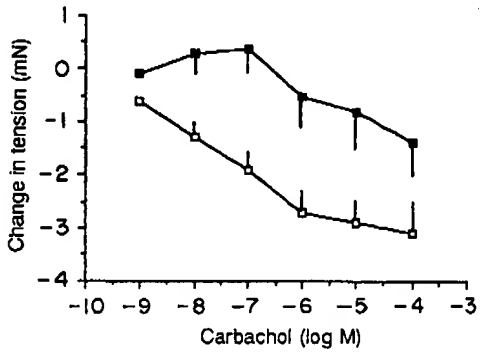

FIG. 6. The effect of DETC (5.0 mM) pretreatment (30 min) *ex vivo* on the tension changes following CCh treatment in Precon-PH vaccinated rats. Vascular endothelium was removed in these experiments. Each point is expressed as mN tension change and presented as the mean ± SEM (n = 4–9). —■—, Precon; —□—, Precon & DETC.

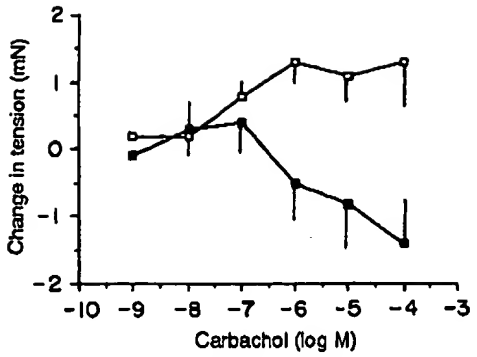

FIG. 7. The effect of MeB pretreatment (10 μM) *ex vivo* on the tension changes following CCh treatment in Precon-PH vaccinated rats. Vascular endothelium was removed in these experiments. Each point is expressed as mN tension change and presented as the mean ± SEM (n = 4–9). —■—, Precon; —□—, Precon & MeB.

intact vessels through the release of nitric oxide (Amezcua *et al.*, 1988), such an impairment suggested that vaccination may either alter the synthesis of nitric oxide or the smooth muscle response to nitric oxide. Such an impairment suggests fundamental differences in the response to vaccination as compared to endotoxaemia (Julou-Schaeffer *et al.*, 1991). Furthermore, both vaccines convert the normal CCh-mediated contractile responses of endothelial-denuded vessels into a relaxant response. This observation suggests that vaccination causes metabolic disturbances in vascular endothelium as well as vascular smooth muscle. These studies utilized biophysical responses to CCh as the end-point. Such responses are a sum of the direct smooth muscle effects of CCh and the indirect effects via release of endothelial factors. Since both vaccines impaired endothelial-dependent relaxations while reversing the smooth muscle contractile responses to CCh into relaxations, it seems reasonable to propose that the endothelium is metabolically altered and may be releasing contractile substances. Scanning electron microscopic examination of the endothelial surface suggests deposition of a fibrin-like material and adhesion or entrapment of circulating cells (data not shown). An alternative possibility is that adherent cells (e.g. neutrophils) may be releasing vasoactive mediators in response to CCh. Indeed, Paulsen *et al.* (1990) reported that *P. haemolytica* lipopolysaccharide stimulated neutrophil adherence to vascular endothelium. The relaxant response of endothelial-denuded vessels following vaccination is antagonized by MeB, an inhibitor of soluble guanylate cyclase (Beasley *et al.*, 1991). Antagonism of the relaxant response by MeB is greater in Precon-PH vaccinated rats, suggesting that the second messenger coupling may be altered differently by the two vaccines. Further evidence for a disturbance in the guanylate cyclase second messenger coupling in vascular smooth muscle is evident in the fact that the relaxant response to hydrogen peroxide is converted into a contraction following vaccination. Some previous work suggests that the relaxation of pulmonary artery in response to hydrogen peroxide is mediated via guanylate cyclase (Burke & Wolin, 1987). Nitric oxide, a metabolite of L-arginine which is synthesized in both endothelium and vascular smooth muscle (Schini & Vanhoutte, 1991), exerts its vasoactive effects *via* stimulation of guanylate cyclase (Rapoport & Murad, 1983). Endothelial-denuded vascular smooth muscle relaxes to L-arginine (Schini & Vanhoutte, 1991), suggesting that the smooth muscle contains an L-arginine-nitric oxide pathway independent of the endothelium. Further, bacterial lipopolysaccharide has been associated with activation of the L-arginine–nitric oxide pathway in vascular smooth muscle (Julou-Schaeffer *et al.*, 1991). However, incubation of vascular smooth muscle from control rats and and rats vaccinated with L-arginine did not alter tension. On the other hand, incubation with L-NMMA, an antagonist of nitric oxide synthesis, increased the magnitude of the relaxant response in vaccinates. Indeed, L-NMMA is capable of generating vasoactive compounds in vascular smooth muscle (Thomas & Ramwell, 1992) and it is possible that vaccination enhances such a biochemical pathway.

The inhibition of superoxide dismutase with DETC increases the relaxant response to Precon-PH, suggesting that superoxide anion may be involved in that effect; however, DETC treatment inhibited the relaxation response to CCh in vessels from Presponse vaccinated animals. These observations suggest that superoxide anion may be acting as a

vasoconstrictor in vessels from Presponse vaccinated rats while acting as a vasorelaxant in vessels from Precon-PH vaccinated rats. Such vasoactive properties may be direct or indirect (e.g. inactivation of nitric oxide), indicating some difference in the response of host vasculature to these two antigenically distinct vaccines. Indeed, Presponse probably contains adjuvants which would enhance the immunological events associated with antigen processing, whereas Precon-PH is a live 'avirulent' bacterium. Previous studies have shown that superoxide anion is a vasorelaxant in several vascular beds (Lamb & Webb, 1984; Kontos, 1985; Wolin & Belloni, 1985). The mechanism by which the effect of superoxide anion may be differentially altered by vaccination may relate to the pattern of cytokines released. For example, tumour necrosis factor (TNF) has been shown to act as a free radical scavenger *in vitro* (Matsubure *et al.*, 1991), and both TNF and interleukin-1 protect the lung against oxygen toxicity (Berg *et al.*, 1990). Indeed, treatment with interleukin-2 has been shown to impair *in vivo* relaxant responses of rat cerebral arteries to acetylcholine (Ellison *et al.*, 1990).

In summary, these experiments demonstrate that vaccination with two antigenically distinct *Pasteurella* vaccines alters endothelial-dependent and -independent changes in vascular smooth muscle responses to CCh. The experiments suggest that multiple biochemical pathways may be altered by vaccination. Similar circulatory disturbances occurring *in vivo* during the immediate post-vaccination period might impair host defenses and increase susceptibility to disease.

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